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Integrative indices for health assessment in reef corals under thermal stress

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ABSTRACT

Global warming is one of the major causes of reef coral ecosystems' degradation. Predictions of further rise in sea surface temperatures call for urgent action. In this study, a holistic method for bio-monitoring heat stress in reef ecosystems was tested and optimized. Long-term induction of antioxidant enzymes and oxidative stress by elevated temperatures (30 °C and 32 °C) was assessed on fragments of reef-building corals and compared to control conditions (26 °C). The quantification of both oxidative stress, through lipid peroxidation (LPO) levels, and antioxidant enzyme activities: superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) in a long-term experiment (60 days), using seven Indo-Pacific reef-building coral species, provided useful information that was interpreted in combination with the observed partial mortality and growth rate of these organisms. These biomarkers were combined in integrated biomarker response (IBR) indices, either in an antioxidant defense mechanisms and oxidative stress response category (approach A: GST, CAT, LPO, and SOD) or in an integrated stress response category – organism performance (approach B: GST, CAT, LPO, SOD, partial mortality, and growth rate). The results of this study indicate that the IBRs were responsive to temperature treatment and dependent on the coral species. The approach B was the most adequate since it better reflected the stress suffered by the tested species, whereas the set of four biochemical biomarkers (approach A) was not enough to explain the organismal response of most of the tested species to thermal stress conditions.

1. Introduction

Global warming has profound implications upon marine ecosystems (Hoegh-Guldberg et al., 2007; Hughes et al., 2003). Thermal stress affects all physiological processes, ranging from protein damage to organism performance (Hochachka and Somero, 2002). Ultimately, it can lead to mass mortality events, an issue that has been particularly serious in many coral reefs at a worldwide level, raising public and scientific concern (Fabricius et al., 2007; Hoegh-Guldberg et al., 2007). As reef-building corals are habitat-forming species that strongly influence the macro- and micro- structure of the habitat (Dayton, 1972), ecosystem functions may be severely affected by their degradation.

Most reef-building corals may not have the ability to fast adapt to the accelerating pace of climate change (Skelly et al., 2007). Episodes of mass coral bleaching in the Indo-Pacific region have resulted in the replacement of dominant and branching heat-susceptible coral species by sub-dominant and massive heat-tolerant coral species in some locations (Van Woesik et al., 2011), and in shifts in dominance from reef-building corals to macroalgae or other sessile invertebrate-dominated benthic functional groups in many others (Hughes et al., 2010; Norström et al., 2009; Nyström et al., 2000; Tebbett et al., 2019). Therefore, mass bleaching episodes have resulted in an overall decline of biodiversity and loss of functional complexity (Ateweberhan et al., 2011; Baker et al., 2008; Graham et al., 2008; Pratchett et al., 2008).

Average sea surface temperatures (SSTs) near coral reef ecosystems are predicted to further rise 1–3.7 °C over the 21st century (IPCC, 2014, 2018). Therefore, it has been suggested that, unless there is considerable thermal adaptation of both coral hosts and their symbiotic algae (Baird and Maynard, 2008; Donner, 2009; Donner et al., 2005), mass bleaching and mortality events could occur annually on the world's coral reefs by 2050 (Nicholls et al., 2007; Van Hooidonk and Huber, 2009). In consequence of global warming, extreme events (e.g. tropical storms) are predicted to increase in severity and frequency, leading to a shorter time for recovery between recurrences (Elsner et al., 2006;

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Emanuel, 2005; IPCC, 2014) and increase in the physical damage experienced by coral reefs (Hoegh-Guldberg, 2011).

The maintenance, repopulation, and recovery of reef coral communities after disturbance are highly dependent on both sexual and asexual reproductive processes (Connell and Keough, 1985; Glynn et al., 2017). The ratio of sexual and asexual recruitment is expected to change over the geographic range of a species, depending on the frequency of sexual recruitment, genet longevity (Coffroth and Lasker, 1998), and disturbance effects (e.g. ocean warming and tropical storms; Baums et al., 2006). Studies have shown that heat stress can negatively impact all the stages of corals' sexual reproductive life cycle (e.g. Heyward and Negri, 2010; Levitan et al., 2014; Negri et al., 2007; Nozawa and Harrison, 2007; Randall and Szmant, 2009). Therefore, the recurrent increases in SSTs will likely lead to the failure of sexual reproduction, being asexual propagation the most probable mean of natural recovery under current changes in climatic conditions.

Tropical storms are favorable to the propagation and expansion of scleractinian corals of various morphologies by asexual reproduction of storm-generated fragments through the reefs (Foster et al., 2007, 2013; Highsmith, 1980, 1982; Highsmith et al., 1980; Lirman, 2000; Tunnicliffe, 1981), which later re-attach to the substrate, grow and form a new colony (Foster et al., 2007). Fragmentation is considered an adaptation to both favorable and unfavorable environmental conditions (Honnay and Bossuyt, 2005; Lirman, 2000). Although coral fragmentation alone may not be the solution for coral reef recovery, this asexual mode of reproduction allows coral species to persist disturbance when they cannot complete their sexual reproductive life cycle. Given the future projections of increase in tropical storms' intensity and frequency, asexual reproduction may increase through fragmentation.

In a changing climate, ecosystems where foundation species are susceptible to the effects of elevated temperatures are thus vulnerable to major reorganization, being characterized by reduced habitat complexity and disrupted ecosystem services (Ellison et al., 2005; Hoegh-Guldberg and Bruno, 2010). Recent approaches of assessing global warming impacts focus on quantifying exposure to climatic change, mostly disregarding the biological differences between species that significantly affect their vulnerability (Harley et al., 2006). The diversity of responses to environmental change among species contributing to the same ecosystem function (i.e. response diversity) is critical to resilience (Elmqvist et al., 2003). Response diversity is particularly important for ecosystem renewal and reorganization following disturbance, providing adaptive capacity in a world subjected to extreme climatic events.

The urgent need to assess the quality of marine ecosystems led to the development of several monitoring tools (Devin et al., 2014). Changes in community structure and measures of chemical contamination are frequently used to indicate ecosystem health status (Chase et al., 2001; Dustan and Halas, 1987; Hughes, 1994; Viarengo et al., 2000). Nevertheless, these represent damage manifestations rather than prognostic indices (Knap et al., 2002). Biomarkers are an example of responses that have provided valuable mechanistic information to scientists, allowing environmental managers' action before ecosystem health deterioration has occurred (Lam, 2009; Marigómez et al., 2013). Nonetheless, the multi-biomarker approaches are generally hard to interpret, and produce results that are not easy to integrate in the environmental policies framework (Beliaeff and Burgeot, 2002). To fill this gap, integrative indices have been developed, and one of the most used is the Integrated Biomarker Response (IBR) (Beliaeff and Burgeot, 2002). The IBR indices have been applied for monitoring and rank the effects of chemical contamination (e.g. Broeg and Lehtonen, 2006; Marigómez et al., 2013; Devin et al., 2014; Lehtonen et al., 2014). These indices have also been applied in a climate change context (Madeira et al., 2016, 2018; Maulvault et al., 2018, 2019; Marques et al., 2019).

The integration of biomarkers in health indices may provide comprehensive information about the biological effects of environmental variables in marine organisms (Marigómez et al., 2013) and may thus serve as a valuable tool for environmental managers (Broeg and Lehtonen, 2006; Madeira et al., 2018). This tool can be combined with morphological assessments to characterize the sub-lethal metabolic effects of general stressors in marine organisms. Madeira et al. (2018) defined and tested a holistic method for bio-monitoring based on a set of biomarkers in order to evaluate the effects of ocean warming in selected tropical fish, crustaceans, and gastropods. Nevertheless, no similar approach has been applied and tested in reef-building coral species. The definition of an adequate set of biomarkers may be crucial for more precise predictions of environmental health in tropical marine environments, which are urgently needed given the expected increases in SSTs and associated extreme events.

Dias et al. (2018, 2019a) investigated the partial mortality, growth rate, antioxidant enzyme activities and oxidative stress on small fragments of nine Indo-Pacific reef-forming coral species exposed for 60 days to increasing temperatures (30 °C and 32 °C) and compared results with control temperature (26 °C), mimicking a post-storm scenario in a warming of tropical oceans context. These studies identified different susceptibility among coral species to conditions that are predicted to be more frequent in the future, influencing coral asexual reproduction via fragmentation. In Dias et al. (2018), partial mortality was assessed every 20 days of experiment, whereas growth rate was assessed on the last day of experiment. Furthermore, in Dias et al. (2019a), lipid peroxidation (LPO) levels and the activities of catalase (CAT) and glutathione S-transferase (GST) were quantified on the last day of experiment. The present study used the same experimental procedure and data obtained in Dias et al. (2018, 2019a), with the exception of SOD activity data that were not included in Dias et al. (2019a) and are presented here for the first time.

The present study aims to test and optimize two different integrated biomarker approaches to be applied in the monitoring of the effects of heat stress generated by global climate change in the Indo-Pacific region. The two integrated biomarker approaches were divided in Approach A – antioxidant defense mechanisms and oxidative stress response category: based on a set of four biochemical biomarkers (GST, CAT, LPO, and SOD) and Approach B - integrated stress response category (organism performance): based on the combination of four biochemical biomarkers and two biomarkers at organismic level (GST, CAT, LPO, SOD, partial mortality, and growth rate, respectively) (Cooper et al., 2009; Monserrat et al., 2007). First, the two approaches were applied to small fragments of seven reef-building coral species of the Indo-Pacific region exposed to both control and two stress temperatures. Second, integrated biomarker response (IBR) index values were obtained for each species and temperature treatment combination within the two different approaches. Finally, the advantages and disadvantages of the two approaches were compared and discussed.

2. Materials and methods

2.1. Study species

This study included seven common and widely distributed reef-building coral species of the Indo-Pacific region (Veron, 1990, 2000) with four different morphologies: one massive species (*Galaxea fascicularis*), one encrusting species (*Montipora capricornis* green morphotype (GM)), three plating species (*Montipora capricornis* brown morphotype (BM), *Echinopora lamellosa*, and *Turbinaria reniformis*), and two branching species (*Acropora tenuis* and *Psammocora contigua*). Information relative to the growth rate and partial mortality of the tested coral species was taken from the literature and given in Table 1. These coral species were chosen in order to use the largest number of species available at "Oceanário de Lisboa" (a large public aquarium, www.oceanario.pt) with different i) colony morphology, a characteristic mentioned as having influence in coral species susceptibility to heat stress (Loya et al., 2001) and ii) heat stress susceptibility: severe (*A.*

Table 1
Published data of growth rate given in four metrics - surface area ($cm^2.year^{-1}$), linear extension ($mm.year^{-1}$), relative growth rate (% day^{-1}) and specific growth rate (day^{-1}) - and partial mortality for the tested coral species. No data were found for *Montipora capricornis* (both morphotypes) and *Psammocora contigua* species.

Coral species	Growth rate				Partial mortality (%)	Reference
	Surface area ($cm^2.year^{-1}$)	Linear extension ($mm.year^{-1}$)	Relative Growth Rate (% day^{-1})	Specific Growth Rate (day^{-1})		
Acropora tenuis	–	–	0.82–1.24	–	–	Anticamara and Tan, 2018
	–	5.6	–	–	–	Mahmoud et al., 2019
	–	17.2–30.7	–	–	–	Nakamura et al., 2011
	–	29.6–64.2	–	–	0.0	Rocker et al., 2017
Echinopora lamellosa	–	–	0.50–0.73	–	–	Anticamara and Tan, 2018
	11.40–18.72	–	–	–	–	Dela Cruz et al., 2015
	4.44–6.24	–	2.60–3.60	–	–	Levy et al., 2010
	4.31–8.58	–	0.62–0.87	–	–	Shaish et al., 2008
	–	–	0.01–0.14	–	0.7–43.1	Shaish et al., 2010
Turbinaria reniformis	–	–	0.08–0.10	–	–	Ezzat et al., 2016
	–	–	0.78	–	–	Orejas et al., 2011
	–	5.6–15.3	–	–	–	Ross et al., 2018
	–	–	0.48	–	–	Tolosa et al., 2011
Galaxea fascicularis	–	–	0.04	–	–	Anticamara and Tan, 2018
	–	–	0.15–0.49	–	–	Bhagooli and Hidaka, 2002
	–	26.0	–	–	–	Ngai et al., 2013
	–	–	0.14	–	–	Orejas et al., 2011
	–	–	–	0.014	–	Schutter et al., 2008
	–	–	–	0.012	–	Schutter et al., 2010
	–	–	–	0.030	–	Wijgerde et al., 2012

tenuis), high (*M. capricornis*), moderate (*E. lamellosa*), and low (*T. reniformis*, *G. fascicularis*, and *P. contigua*) (Dias et al., 2018; Marshall and Baird, 2000). Coral species were identified according to Veron (2000). The coral colonies used in the experimental treatments have been maintained in captivity in a coral stock aquarium at "Oceanário de Lisboa (Portugal)" for five years, providing their thermal history information.

2.2. Experimental procedure

The experiment was conducted from April to November of 2014 at "Oceanário de Lisboa, Portugal" (www.oceanario.pt). Ten replicate fragments were cut from each coral colony, using a pincer or a pair of pliers. For the branching colonies the fragments were cut approximately 20–40 mm in length and the fragments for the massive, plating, and encrusting colonies were obtained by cutting approximately 30 mm sided squares. All fragments were placed over egg crate panels in the coral stock aquarium until acclimation to the experimental aquarium.

The live wet mass of each coral fragment was obtained by blotting it with a paper towel to remove excess seawater, then weighing it in air on an electronic balance to the nearest 0.01 g (Titlyanov et al., 2005). Each fragment was glued with epoxy putty to the top of a pre-weighed and numbered nylon expansion anchor. Placement of the fragment varied by morphology with the branching fragments in vertical position and the plating, encrusting and massive fragments placed in horizontal positions. Then, the set (coral fragment + anchor) was weighed to remove the epoxy putty weight off the calculations and placed back over egg crate panels in the coral stock aquarium, to recover from the handling procedures for one day before acclimation to the experimental aquarium.

To determine the response of the seven-coral species to the effect of increased temperatures, the coral fragments were exposed to different treatments: (a) control temperature (26 °C) (26.1 ± 0.2 SD) and (b) increased temperatures (30 °C and 32 °C) (30.2 ± 0.5 SD and 32.2 ± 0.5 SD, respectively). Ten coral fragments of each coral species were exposed to each of the different treatments for 60 days. Coral fragments were acclimated 1 h per °C above the temperature of the coral stock aquarium (25 °C). This heating rate was applied because coral reef-flat communities can experience temperature changes of 1 °C $hour^{-1}$ during spring tides (Berkelmans and Willis, 1999), and most of the coral species in this study colonize the reef-flat zone (Brown and Suharsono, 1990; Fujioka, 1998). The coral fragments were placed 2 cm apart from one another and arranged by coral species.

The experimental aquarium (400 L) was fitted with a sump (280 L) filled with bioballs for biological filtration in which two Fluval M300 heaters, as well as a Hailea 500 chiller controlled water temperature. For water circulation purposes, an AquaMedic OceanRunner 3500 pump provided a turnover rate of 5 times per hour. An AquaMedic Turboflotor 5000 Shorty protein skimmer helped keeping nutrient concentrations low and increased surface water motion in the aquarium was accomplished by using an AquaClear 110 powerhead. Lighting requirements similar to the coral stock aquarium were attained by using a Litpa Jet5 floodlight with an AquaMedic 400 W HQi lamp (13,000 K) on a 12 h light/ 12 h dark cycle. An air-stone was used in the aquarium to ensure good oxygen concentrations.

Photosynthetically Active Radiation (PAR) levels were measured with a spheric quantic sensor (LI-193SA) and a data logger (1400 model) and varied between 320 and 345 $\mu E.m^{-2}\ s^{-1}$ in the 400–700 nm waveband. Water quality parameters such as water temperature, pH and salinity were measured on a daily basis. Water samples were also weekly analyzed to determine ammonium, nitrites, nitrates, calcium concentration as well as oxygen concentration and saturation and alkalinity. These parameters were maintained as follow: pH at 8.2–8.3, salinity at 33–33.5 psu, alkalinity approximately at 100 mg l^{-1}, nitrites between 0.002 and 0.005 mg l^{-1}, nitrates between 0 and 2 mg l^{-1}, calcium concentration between 389 and 401 mg l^{-1}, oxygen saturation at 104% and oxygen concentration between 6.5 and 7.1 mg l^{-1}. Salinity was maintained with daily balanced additions of reverse osmosis freshwater and filtered artificial seawater. Whenever alkalinity levels were below 100 mg l^{-1} sodium bicarbonate was added to the system. Aquarium cleaning routines were done as required to avoid algal growth, and these included expansion anchors cleaning with a toothbrush and egg crate replacement, at least 3 times a week.

2.3. Analytical procedures

2.3.1. Partial mortality assessment

Partial mortality was visually quantified by estimating the percentage of dead area in ten fragments of each coral species per temperature treatment on the 60th day of experiment.

2.3.2. Growth rate measurements

The coral fragments were weighted both in the first and last day of the experiment in order to calculate the growth rates for each coral fragment, except for the dead ones. The relative growth rate (RGR), expressed as daily biomass increase, was calculated using the formula:

$$RGR = [\ln(Wf/Wi)]/[(tf - ti)] \times 100$$

where Wi is the initial weight, Wf is the final weight and tf and ti is the time interval between the starting and end date (Marinho-Soriano et al., 2006).

2.3.3. Samples collection and storage

Six fragments of each coral species per treatment were removed from the experimental aquarium, separated from the respective anchor, and placed inside individual and identified sterilized bottles on ice-cold conditions. Nevertheless, there were combinations of experimental treatments and coral species where no fragments were taken at all due to mortality. In the temperature treatments where the coral species were alive, the coral fragments with the highest amount of tissue were selected, this is, the coral fragments presenting the lowest or no partial mortality at all, since the ones presenting high partial mortality would not have enough tissue for biomarker analysis. The number of fragments of each coral species used in the four biochemical biomarkers' analysis per treatment is given in supplementary material (SM1). Then, they were kept inside refrigerated boxes and transported to the laboratory where they were stored at −80 °C.

2.4. Antioxidant defense enzymes and lipid peroxidation assays

2.4.1. Protein extraction

Coral fragments were carefully rinsed with ultrapure water to remove saltwater and any debris and then the excess moisture was dried with absorbent lab paper. Afterwards, all the coral fragments were smashed (1 fragment = 1 sample) with a mortar and pestle and then were placed on 5 mL plastic microtubes. The samples were then homogenized in 1 mL of phosphate buffered saline solution (PBS consisting of 0.14 M NaCl, 0.003 M KCl, 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4, pH 7.4) to extract cytosolic proteins, using a glass/Teflon Potter Elve-jhem tissue grinder, in ice-cold conditions and mixed. The crude homogenates were then centrifuged at 4 °C for 15 min at 10,000 × *g*. The supernatant was collected, transferred to new microtubes (1.5 mL) and frozen immediately (−80 °C).

2.4.2. Total protein determination

For normalizing the results, the total protein content was determined through the Bradford method (Bradford, 1976). A calibration curve was obtained using bovine serum albumin (BSA) standards (0–2.0 $mg.mL^{-1}$).

2.4.3. Oxidative damage products – Lipid peroxidation

The lipid peroxides assay was adapted from the thiobarbituric acid reactive substances (TBARS) protocol (Uchiyama and Mihara, 1978). In brief, five μL of each sample, already processed as previously described were added to 45 μL of 50 mM monobasic sodium phosphate buffer. Then 12.5 μL of SDS 8.1%, 93.5 μL of trichloroacetic acid (20%, pH = 3.5) and 93.5 μL of thiobarbituric acid (1%) were added to each microtube. To this mixture, 50.5 μL of Milli-Q grade ultrapure water was added. Then, the microtubes were put in a vortex for 30 s, centrifuged at 10,000 × *g* for 1 min, their lids were punctured with a needle and then incubated in boiling water for 10 min. To stop the reaction, they were placed on ice for a few minutes and 62.5 μL of MilliQ grade ultrapure water was added. Then, the microtubes were placed in a vortex and centrifuged at 10,000 × *g* for 1 min. Duplicates of 150 μL of the supernatant of each reaction were put into a 96-well microplate and absorbance was read at 530 nm. To quantify the lipid peroxides, an eight-point calibration curve (0–0.3 μM TBARS) was calculated using malondialdehyde bis (dimethylacetal) (MDA) standards (Merck Millipore, Portugal).

2.4.4. Enzymatic assays

2.4.4.1. Superoxide dismutase activity. Superoxide dismutase (SOD) activity (EC1.15.1.1) was assessed by using nitroblue tetrazolium (NBT) and xanthine oxidase (XOD) according to Sun et al. (1988). After reading the absorbance at 560 nm, SOD activity was calculated using the following equation for the % inhibition:

((Abs$_{560}$ / min negative control – Abs$_{560}$ / min sample) / (Abs$_{560}$ / min negative control)) × 100

2.4.4.2. Catalase activity. Catalase (CAT) activity (EC1.11.1.6) was assessed by using the peroxidatic function of catalase for determination of enzyme activity. The method is based on the reaction of catalase with methanol in the presence of hydrogen peroxide according to a method previously described by Johansson and Borg (1988) and adapted for 96-well microplate. In brief, 20 μL of sample in sample buffer (25 mM of KH_2PO_4, containing 1 mM EDTA and 0.1% BSA; pH 7.0), 30 μL methanol and 100 μL of assay buffer (100 mM KH_2PO_4, pH 7.0) were added to microplate wells. Then, 20 μL of standard (4.25 mM formaldehyde), 30 μL methanol and 100 μL of assay buffer were added to formaldehyde standard wells. To the positive control wells, were added 20 μL of diluted CAT (bovine liver CAT), 30 μL of methanol and 100 μL of assay buffer. Afterwards, the reaction was initiated by adding 20 μL of hydrogen peroxide (0.035 M H_2O_2) to the microplate wells. Then, the microplate was covered with aluminum foil and incubated during 20 min at room temperature under gentle agitation on a shaker. Following incubation, 30 μL of potassium hydroxide (10 M KOH) was added to each microplate well to end the reaction, followed by adding 30 μL of Purpald chromogen (34.2 mM of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) to each well. Again, the microplate was covered and incubated on a shaker for 10 min at room temperature. Next, 10 μL of potassium periodate (65.2 mM KIO_4) was added to each well and the microplate incubated for 5 min on the shaker. The absorbance was read at 540 nm using a microplate reader (Benchmark, Bio-Rad, USA).

2.4.4.3. Glutathione S-transferase activity. The enzymatic assay of glutathione S-transferase (GST) activity (EC 2.5.1.18) was adapted from Habig et al. (1974), using the CDNB (1- chloro-2,4-dinitrobenzene) as the enzyme substrate, and optimized for 96-well microplates. After reading the absorbance at 340 nm GST activity was calculated using a molar extinction coefficient for CDNB of 5.3 εmM (mM^{-1} cm^{-1}) after correction for the microplate wells path length.

2.5. Data analysis

Only the individual coral fragments used in the antioxidant defense enzymes and lipid peroxidation assays per species and temperature treatment were used in data analysis (SM1). In order to integrate all results from different biomarkers (biochemical and organismic levels), the integrated biomarker response (IBR) was calculated. The IBR, a simple multivariate graphic method – star plot – was calculated according to Beliaeff and Burgeot (2002) to allow visual integration of a set of early warning responses measured with biomarkers. To calculate the IBR for biomarkers, the general mean (*m*) and the standard deviation (*s*) of all data regarding a given biomarker were calculated, followed by a standardization for each situation to obtain *Y*, where $Y = (X$

- $m)/s$, and X is the mean value for the biomarker at a given species and temperature treatment interaction. Then Z was calculated using $Z = -Y$ or $Z = Y$, in the case of a biological effect corresponding to inhibition or activation, respectively. Concerning the biological effect considered for each parameter, growth rate was assumed to decrease upon temperature increase. In a similar way, the antioxidant enzyme activities (SOD, CAT, and GST), oxidative stress (LPO), and partial mortality were always assumed to increase with the exposure to increased temperatures. The score (S) was calculated by $S = Z + |Min|$, where $S \geq 0$ and $|Min|$ is the absolute value for the minimum value for all calculated Y in a given biomarker. Note that in the cases where –Y is applied the minimum also changes, as all the distribution does. Star plots were then used to display Score results (S) and to calculate the IBR as follows:

$IBR = \sum_{t=1}^{n} A_i$, being

$A_i = \frac{Si}{2} \sin\beta\ (S_i \cos\beta + S_{i+1} \sin\beta)$

and

$\beta = \tan^{-1}((S_{i+1}\sin(\alpha))/(S_i - S_{i+1}\cos(\alpha)))$, and where S_i and S_{i+1} are two consecutive clockwise scores (radius coordinates) of a given star plot; Ai corresponds to the area connecting two scores; n the number of biomarkers used for calculations; and $\alpha = 2\pi/n$. The IBR is obtained by summing up all the Ai. The IBR calculations were always performed with the same order of parameters for all species and temperature interactions: the biochemical biomarkers GST, CAT, LPO and SOD, followed by partial mortality and growth rate.

Biomarkers were divided in two stress response categories:

i) Approach A – Antioxidant defense mechanisms and oxidative stress response category: based on a set of four biochemical biomarkers (GST, CAT, LPO, and SOD) and ii) Approach B - integrated stress response category (organism performance): based on the combination of four biochemical biomarkers and two biomarkers at organismic level (GST, CAT, LPO, SOD, partial mortality, and growth rate, respectively) - for the index calculations. To evaluate patterns in the biomarker variations, the variables were standardized (to each sample value the mean was subtracted and then divided by the standard deviation of all samples).

The Sensibility (Sb) of coral species to heat stress, expressed as %, was calculated as $Sb = ((IBR_2 - IBR_1)/IBR_1) \times 100$; where IBR_2 is the value of IBR at the testing temperatures conditions (i.e. 30 °C or 32 °C) and IBR_1 is the value of IBR in the control treatment (26 °C).

Additionally, we also analyzed biomarker scores as a fitness index (E), calculated as $E = (S_1 - S_2)$ (Ferreira et al., 2015), where E denotes effect and S_1 and S_2 stand for the scores of two different treatments. E was calculated for all possible combinations where S_1 always corresponded to the scores of a lower temperature treatment than S_2. Values that differed in 0.5 points from S_1 were considered to be from an animal with a higher or lower fitness (higher or lower scores, respectively) (Ferreira et al., 2015).

3. Results

3.1. IBR approach A – Antioxidant defense mechanisms and oxidative stress response category

In general, IBR values increased with increase in temperature (Fig. 1a–d and f). Exceptions to this pattern were *Turbinaria reniformis,* that presented a decrease of IBR values from 26 °C to 30 °C, but an increase in IBR values from 30 °C to 32 °C (Fig. 1e), and *Psammocora contigua* that presented a decrease of IBR values with increase in temperature (Fig. 1g). Regarding the sensibility of the coral species to heat stress, the species *M. capricornis* (BM) was the most sensitive species at 30 °C and *G. fascicularis* was the most sensitive species at 32 °C (Table 2).

Regarding the magnitude of differences between temperature treatments, the most responsive biomarkers to elevated temperature were GST, CAT, SOD (especially in *M. capricornis* (BM) and *E. lamellosa*, Fig. 1b and d) and LPO (*E. lamellosa* and *G. fascicularis,* Fig. 1d and f).

3.2. IBR approach B - integrative stress response category (organism performance)

Star plots and index values show that increased temperature did not affect equally all tested species (Fig. 2). *Montipora capricornis* (BM) was the most sensitive species at 30 °C and *G. fascicularis* was the most sensitive species at 32 °C (Table 2).

The most responsive biomarkers (i.e. biomarkers that presented a ≥ 60% variation in the S values among temperature treatments for each species) were LPO, SOD, partial mortality and growth rate in *A. tenuis*; GST, CAT, LPO, SOD and partial mortality in both *M. capricornis* (BM) and *E. lamellosa*; GST, CAT, SOD and partial mortality in *M. capricornis* (GM); SOD, partial mortality and growth rate in *T. reniformis*; GST and LPO in *G. fascicularis*; GST, CAT, LPO, partial mortality and growth rate in *P. contigua*. The markers least influenced by increased temperature (<60% variation in the S values) were growth rate (*M. capricornis* (BM), *M. capricornis* (GM), *E. lamellosa,* and *G. fascicularis*), CAT (*A. tenuis, T. reniformis,* and *G. fascicularis*), SOD (*G. fascicularis* and *P. contigua*), LPO (*M. capricornis* (GM), and *T. reniformis*), and partial mortality (*G. fascicularis*) (Fig. 2).

3.3. Fitness index

Results showed that biomarkers consistently scoring higher at both 32 °C and 30 °C than at 26 °C were also the ones indicating deleterious effects ($E \leq -0.5$, in red in Table 3), namely SOD and growth rate. Biomarkers scoring higher at 32 °C than at 26 °C include growth rate, whereas the ones scoring higher at 32 °C than at 30 °C include SOD. In contrast, biomarkers indicating no detectable effects ($-0.5 < E < 0.5$, in yellow in Table 3) in fitness in the long-term exposure to increased temperature were LPO and CAT for most species (Table 3).

In general, a decrease or maintenance in the fitness index (red filled boxes and yellow filled boxes, respectively) was observed with increase in temperature for most coral species and biomarkers. The species *E. lamellosa* and *T. reniformis* presented an increase in the fitness index ($E \geq 0.5$, in green in Table 3) in one biomarker from 26 °C to 30 °C. The *E. lamellosa* fragments presented it in partial mortality, whereas the fragments of *T. reniformis* presented it in GST (Table 3). The fragments of *P. contigua* presented an increase in the fitness index in partial mortality from 26 °C to 30 °C and from 26 °C to 32 °C. The most responsive biomarkers were growth rate and SOD, showing greater deleterious effects in the fitness index results (Table 3). All biomarkers were affected by temperature, except for LPO (Table 4). The most responsive biomarkers were growth rate > SOD > GST > CAT > PM > LPO.

4. Discussion

In this study, an integrated approach was used for health assessment of reef corals under thermal stress by exposing small fragments of seven common and widely distributed corals species of the Indo-Pacific oceans (Veron, 1990) to both control and two stress temperatures. Our results showed that an increase of 6 °C in SSTs will lead to deleterious effects at two levels of biological organization for most of the tested species. The health status of indicator species within ecosystems can be successfully assessed through multivariate biomarker approaches involving multiple biological and physiological measurements as previously stated by Hook et al. (2014). Therefore, we tested this methodology in coral reefs, and two different comparative approaches were applied: the first containing just the biochemical biomarkers (approach A), whereas the second one contained the biochemical biomarkers and two biomarkers at organismic level (approach B). The chosen biochemical biomarkers, glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), and lipid peroxidation (LPO), were

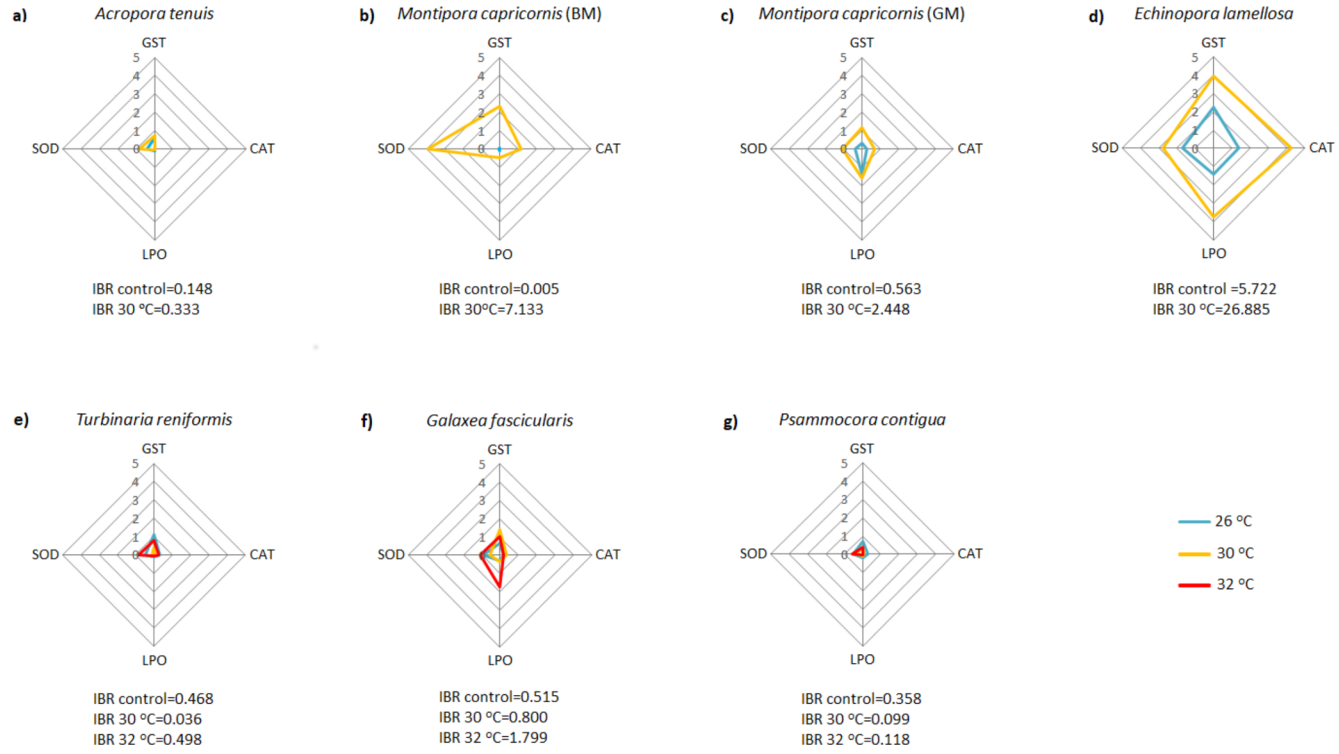

Fig. 1. Star plots with mean scores for the seven coral species exposed to 26 °C (control) and both 30 °C and 32 °C (stress temperatures), the first four coral species (a, b, c, d) did not present values at 32 °C due to total mortality of the fragments. GST – glutathione S-transferase; CAT – catalase; LPO – lipid peroxidation; SOD – superoxide dismutase.

previously used in studies evaluating the effect of heat stress in reef-building coral species (Dias et al., 2019a,b; Downs et al., 2000). Also, growth rate, one of the physiological responses of an organism, has been widely used in marine invertebrates, e.g. bivalves (Widdows et al., 1982) and gastropods (Wo et al., 1999), to provide a measure of environmental quality. Partial mortality has also been applied as an indicator of environmental stress (Nugues and Roberts, 2003). Overall, both IBR approaches suggest that index values were significantly affected by temperature and coral species. Our results confirm the adequacy of using IBR indices for monitoring physiological responses in populations of tropical reef-building corals that might be induced by heat stress events when the approach includes biomarkers at organismic level.

According with the results obtained from the tested species, approach A did not have enough resolution, this is, the set of four biochemical biomarkers was not enough to explain the response of most of the tested species to the increase in temperature. This resulted from the absence of relation between the magnitude of increase in IBR values with heat stress and the biomarkers at organismic level (partial mortality and growth rate). Therefore, approach A proved incomplete as an environmental health index for most of the coral species. On the other hand, approach B included both biochemical biomarkers, which may be used to detect thermal stress before visual or sub-lethal changes are manifested, and biomarkers at organismic level that are a measurement of the deleterious effect per se after the thermal stress has already occurred. Thus, approach B proved adequate since the combination of biomarkers of different levels of biological organization allowed a better understanding of the heat stress suffered by the tested species.

Most coral species presented an increase in IBR values with temperature, expected due to stress increase with temperature (Madeira et al., 2018). In general, a decrease in growth rate and an increase in both antioxidant enzyme activities and partial mortality values were observed with increase in temperature. The increase in antioxidant enzyme activities observed in most of the tested species is expected since increase in temperature leads to increase in metabolic rates and consequent reactive oxygen species (ROS) overproduction. To maintain cell homeostasis and prevent oxidative damage to intracellular macromolecules, such as lipid peroxidation (i.e. disruption of the cellular membrane and the formation of lipid peroxides), cells use a vast number of antioxidant enzymes that convert ROS into less harmful oxygen forms. In eukaryotes, SOD-CAT is one of the main enzymatic antioxidant pathways (Asada, 1999; Halliwell, 2006). Superoxide dismutase and CAT provide the first line of defense against $O_2^{\bullet-}$ and H_2O_2, respectively. These two antioxidant enzymes limit ROS-mediated damage to intracellular macromolecules, however, they are not completely efficient at executing this task (Hayes and McLellan, 1999). Once some of the chemicals produced following the interaction of ROS with intracellular macromolecules are highly reactive, these secondary

Table 2
Sensibility of the seven Indo-Pacific coral species to heat stress calculated as rate of IBR variation and expressed as %. Negative percentages correspond to IBR values decrease with increase in temperature. NA – not available due to complete mortality at 32 °C.

	T (°C)/species	*A. tenuis*	*M. capricornis* (BM)	*M. capricornis* (GM)	*E. lamellosa*	*T. reniformis*	*G. fascicularis*	*P. contigua*
Approach A	30–26	125	142798	335	370	−92	55	−72
	32–26	NA	NA	NA	NA	7	249	−67
Approach B	30–26	1209	3377	1053	233	−24	138	−52
	32–26	NA	NA	NA	NA	94	147	−51

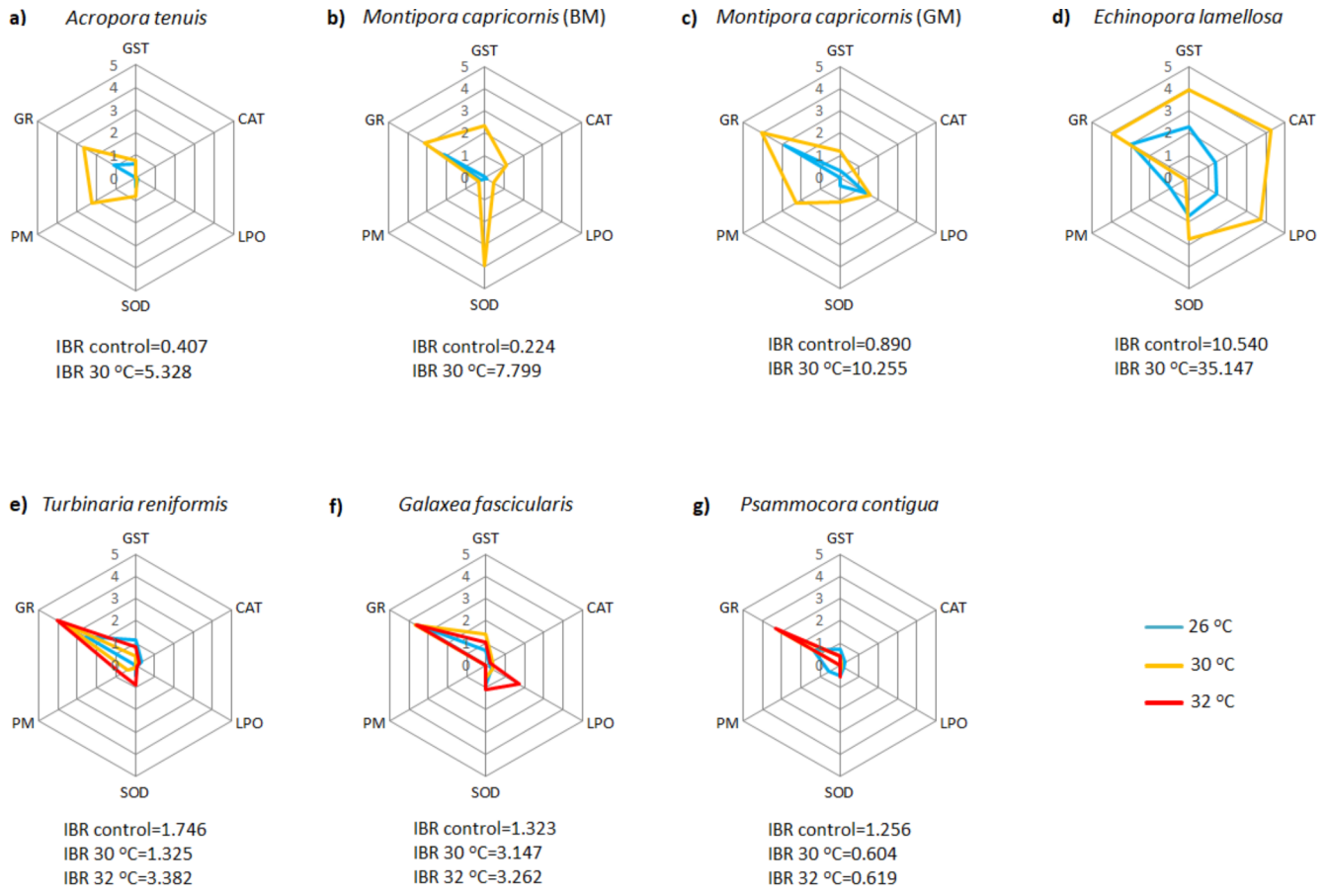

Fig. 2. Star plots with mean scores for the seven coral species exposed to 26 °C (control) and both 30 °C and 32 °C (stress temperatures), the first four coral species (a, b, c, d) did not present values at 32 °C due to total mortality of the fragments. GST – glutathione S-transferase; CAT – catalase; LPO – lipid peroxidation; SOD – superoxide dismutase; PM – partial mortality; GR – growth rate.

oxidation products also need to be detoxified in order to prevent them from also damaging lipids, proteins, and DNA and ultimate lead to cell death (Dixon et al., 2010). This second line of defense against ROS is provided by enzymes such as glutathione S-transferase (GST) (Hayes and McLellan, 1999; Weston et al., 2015). The enzyme GST detoxifies lipid peroxides, having a central role in cell macrostructural repair (Hayes and McLellan, 1999; Limón-Pacheco and Gonsebatt, 2009). The increase in LPO levels, oxidative stress, can occur due to: i) antioxidant pathways being overwhelmed during extreme temperature stress, not detoxifying ROS that begin to accumulate and damage cellular membrane lipids (Weis, 2008), and ii) an induction of an antioxidant response being observed, but it is insufficient to handle the high concentrations of ROS produced and the result is damage to cellular membranes (e.g. *M. capricornis* (BM) and *E. lamellosa*) (Palmer et al., 2009). A decrease in LPO levels was also observed with increase in temperature (e.g. *A. tenuis* and *P. contigua*), which may suggest the occurrence of a remodeling process in the lipid composition of

Table 4
Biomarker scores (mean) for all species at different combinations of temperatures. Red boxes denote deleterious effects ($E \leq -0.5$) and yellow boxes denote no detected effects ($-0.5 < E < 0.5$). GST – glutathione S-transferase; CAT – catalase; LPO – lipid peroxidation; SOD – superoxide dismutase; PM – partial mortality; GR – growth rate.

T(°C)/Biomarker	GST	CAT	LPO	SOD	PM	GR
26-30	-0.669	-0.585	-0.394	-0.740	-0.527	-1.128
26-32	0.076	0.099	-0.408	-0.214	-0.062	-1.191
30-32	-0.014	0.002	-0.444	-0.501	-0.125	-0.143

Table 3
Fitness index presented as a color hitmap: IBR scores were compared for biomarkers between control organisms and those exposed to 30 °C and 32 °C. Red boxes denote deleterious effects ($E \leq -0.5$), green denotes positive effects ($E \geq 0.5$), whereas yellow denotes no detected effect ($-0.5 < E < 0.5$). GST – glutathione S-transferase; CAT – catalase; LPO – lipid peroxidation; SOD – superoxide dismutase; PM – partial mortality; GR – growth rate. NA – not available due to total mortality of the species fragments at 32 °C.

	GST			CAT			LPO			SOD			PM			GR		
Species/T (°C)	26-30	26-32	30-32	26-30	26-32	30-32	26-30	26-32	30-32	26-30	26-32	30-32	26-30	26-32	30-32	26-30	26-32	30-32
A. tenuis	-0.148	NA	NA	0.000	NA	NA	0.070	NA	NA	-0.424	NA	NA	-2.249	NA	NA	-1.528	NA	NA
M. capricornis (BM)	-2.271	NA	NA	-1.128	NA	NA	-0.383	NA	NA	-3.883	NA	NA	-0.125	NA	NA	-1.037	NA	NA
M. capricornis (GM)	-0.851	NA	NA	-0.413	NA	NA	-0.249	NA	NA	-0.680	NA	NA	-2.249	NA	NA	-1.180	NA	NA
E. lamellosa	-1.681	NA	NA	-2.846	NA	NA	-2.307	NA	NA	-1.056	NA	NA	0.750	NA	NA	-1.006	NA	NA
T. reniformis	0.697	0.303	-0.394	0.261	0.074	-0.187	0.037	0.011	-0.026	0.363	-0.416	-0.779	-0.375	-0.750	-0.375	-1.271	-1.538	-0.267
G. fascicularis	-0.750	-0.399	0.351	-0.176	-0.034	0.141	-0.054	-1.413	-1.358	0.342	-0.191	-0.533	0.000	0.000	0.000	-0.083	-0.040	0.042
P. contigua	0.324	0.324	0.000	0.205	0.257	0.052	0.125	0.177	0.052	0.156	-0.034	-0.190	0.562	0.562	0.000	-1.790	-1.993	-0.204

biological membranes (Niu and Xiang, 2018). Finally, a maintenance of the LPO levels was also observed with increase in temperature (e.g. *M. capricornis* (GM) and *T. reniformis*). Although the biomarker of oxidative stress LPO has presented different responses according with the species, this is the only biomarker in approach A that relates to the oxidative damage suffered by the fragments of the tested coral species. Other possible biomarkers that could be applied to evaluate thermal stress are ubiquitin and Hsp70, these biomarkers have already been applied in IBR approaches as macromolecular damage biomarkers and were considered more responsive than LPO (Madeira et al., 2018). The decrease in growth rate with increase in temperature is expected since deviations in temperature of only a few degrees from the optimum temperature lead to growth rate reduction (Berkelmans and Willis, 1999). The calcification rate of several reef-building corals peaks at 26 °C (e.g. Abramovitch-Gottlib et al., 2003; Al-Horani, 2005; Jokiel and Coles, 1977; Marshall and Clode, 2004), being the temperature close to optimal for coral metabolism, thus, above or below this temperature reef-building corals' growth rate decreases. The increase in partial mortality is given to acute stress, it may have resulted from the overwhelming of antioxidant pathways during heat stress, which may have caused localized tissue mortality (Lam, 2009). This response can be observed in the fitness index where most of corals presented a decrease or maintenance of the index.

Different coral species presented different response to heat stress at biochemical and organismic levels. As observed in other studies, coral species differed in their cellular physiology and the strategies applied to diminish oxidative stress (Dias et al., 2019a,b; Downs et al., 2000; Krueger et al., 2015; Marangoni et al., 2019), and in both growth rate and partial mortality according to their inherent traits (Dias et al., 2018, 2019c). For instance, *T. reniformis, E. lamellosa*, and *P. contigua* were the only species presenting increase in the fitness index in one of the biomarkers with increase in temperature.

The biomarkers growth rate and SOD were the most responsive showing greater deleterious effects in the fitness index, whereas LPO remained non-responsive to temperature increase. The increase in SOD activity may be related with this antioxidant enzyme be the first one involved in ROS scavenging, acting close to the site of production (Lesser, 2012). On the other hand, growth rate is a physiological response that takes time to be perceptible (Morgan et al., 2001). Nevertheless, given that our experiment had the duration of 60 days, there was enough time for the observation of changes in growth rate. Regarding LPO non-response, it may be related with the observed increase, decrease or maintenance of the LPO levels according with the coral species and temperature treatment interaction.

In spite of some limitations, IBRs may be an effective method to be applied in the health assessment of reef corals under thermal stress. Biochemical biomarkers (e.g. SOD, CAT, GST, and LPO) tend to be more repeatable and predictable and are much more sensitive for identifying organism stress than whole animal responses (Smit et al., 2009), but their capability to predict significant biological effects is limited (Bartell, 2006). On the other hand, biomarkers at the organismic level (e.g. growth rate and partial mortality), although slower to respond and more difficult to detect, provide "integrated" measures of an organism's well-being based on a range of different functional attributes and are often more ecologically relevant. Therefore, the incorporation of biomarkers of different levels of biological organization provides advantages over the application of single-marker approaches once it allows a better understanding of the heat stress response mechanisms. The approach A did not express well what we could assess visually, however, this approach can be a more sensible indication of sub-lethal stress at the molecular level. The approach B is easy to interpret, however, growth rate is a physiological parameter that takes longer to respond than the biochemical biomarkers. Partial mortality is a sign of extreme stress, leading to the death of the organism unless acclimation is achieved or there is enough growth to surpass the loss of tissue (Guest et al., 2011). Thus, approach B is a more generalist approach, gathering different levels of biological complexity. Based on the obtained results, the use of integrated indices describing heat-induced stress as management and research tools is considered a useful approach.

Still, when applying the IBR index in field populations, it is imperative to assure a careful and adequate selection of biomarkers (Broeg and Lehtonen, 2006). The set of biomarkers used in the present study proved to be adequate for reef-building corals as this set had already responded to heat stress in Dias et al. (2018, 2019a,b). Also, ensuring that the targeted colonies are not exposed to other possible stress factors would be favored (Broeg and Lehtonen, 2006). It is also important to have in mind that these biomarkers are also known for responding to other stress factors beyond those associated with global warming, that is the case of xenobiotics (Downs et al., 2006; Rotchell and Ostrander, 2011), salinity (Dias et al., 2019b, 2019c), pH (Manzello, 2010), sedimentation (Browne, 2012), allelopathy (Chadwick and Morrow, 2011; Morrow et al., 2012), and excess of solar radiation in zooxanthellae (Shick et al., 1995; Dahms and Lee, 2010). Thus, IBR index can actually be fit to assess the reef corals' health when exposed to other factors beyond heat stress, being required further work on the response of corals to such stressors.

CRediT authorship contribution statement

Marta Dias: Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Carolina Madeira:** Investigation, Methodology, Writing - review & editing. **Nadia Jogee:** Investigation, Writing - review & editing. **Ana Ferreira:** Writing - review & editing. **Raúl Gouveia:** Methodology, Writing - review & editing. **Henrique Cabral:** Formal analysis, Writing - review & editing, Supervision. **Mário Diniz:** Methodology, Resources, Writing - review & editing. **Catarina Vinagre:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2020.106230.

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